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# The Role of Magnesium Ions in β-Galactosidase-Catalysed Hydrolyses

## STUDIES ON CHARGE AND SHAPE OF THE β-GALACTOPYRANOSYL BINDING SITE

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1. β-D-Galactopyranosyl trimethylammonium bromide is a competitive inhibitor of β-galactosidase, $K_I = 1.4 \pm 0.2$ mM at 25°C. 2. Tetramethylammonium bromide is not an inhibitor ($K_I > 0.2$ M). 3. The kinetics of deactivation of $Mg^{2+}$-saturated, and of inhibitor- and $Mg^{2+}$-saturated, enzyme in 10mM-EDTA are similar. 4. The apparent $K_I$ for the glycosylammonium salt is approx. 2.2mM in the absence of $Mg^{2+}$. 5. It is therefore concluded that $Mg^{2+}$ and the inhibitor bind independently, and that the $Mg^{2+}$ does not act as an electrophilic catalyst. 6. Complexant fluorescence measurements indicate binding of 1 $Mg^{2+}$ ion per 135000-dalton protomer. 7. This stoicheiometry is confirmed by equilibrium dialysis. 8. 1,6-Anhydrogalactopyranose is neither a substrate ($k_{cat.}/K_m < 3 \times 10^{-2}$ M$^{-1}$·s$^{-1}$) nor an inhibitor ($K_I > 0.2$ M). 9. Considerations of conformations available to the cationic inhibitor and to the anhydrogalactose indicate that the substrate is bound with the pyranose ring in a conformation not greatly different from the normal chair (C1) conformation.

The β-galactosidase of *Escherichia coli* has a requirement for $Mg^{2+}$ for maximal activity in the presence of alkali cations (Tenu *et al.*, 1972). This $Mg^{2+}$ can *a priori* either stabilize a favourable conformation of the enzyme, or act as an electrophilic catalyst (Clark *et al.*, 1970). Evidence has been adduced that the mechanism of action of the enzyme involves generation of a galactopyranosyl cation (Sinnott & Souchard, 1973); any electrophilic catalysis must then occur at the atom directly attached to C-1 of the pyranose ring (cf. Sinnott, 1971). The binding of a potential competitive inhibitor with a positive charge at this atom should therefore be partly competitive with that of $Mg^{2+}$ if the latter is acting as an electrophile. Complications caused by acid–base equilibria can be avoided by the use of quaternary ammonium or phosphonium salts, and so we tested the known β-D-galactopyranosyl trimethylammonium bromide (Micheel, 1929) as both substrate and inhibitor.

We also studied the stoicheiometry of the $Mg^{2+}$ binding, determining $Mg^{2+}$ by the enhancement of the fluorescence of the magnesium chelate of 8-hydroxyquinoline-5-sulphonic acid (Petroský, 1966). The dissociation constant of the chelate is approx. 80μM at 25°C and pH 8.0 (Dawson, 1969); the dissociation constant of the $Mg^{2+}$–enzyme is about 0.65μM (Tenu *et al.*, 1972). Under conditions where 8-hydroxyquinoline-5-sulphonic acid concentration is much greater than both the total $Mg^{2+}$ concentration and the dissociation constant of the 8-hydroxyquinoline-5-sulphonic acid–$Mg^{2+}$ complex, every $Mg^{2+}$ will be in a complex, and therefore fluorescence enhancement will be proportional to total $Mg^{2+}$ concentration. If, further, $Mg^{2+}$-free enzyme is added, then, since the enzyme binds $Mg^{2+}$ more tightly than does 8-hydroxyquinoline-5-sulphonic acid, it will at high $Mg^{2+}$ concentrations take up a stoicheiometric quantity of the metal. However, since the binding is not infinitely tight, at low concentration of metal ion less of it will be bound by the enzyme. Therefore a plot of fluorescence enhancement against concentration of metal ion in the presence of enzyme will be an upward curve, asymptotically parallel to, but lower than, the plot obtained in the absence of enzyme. The spacing on the [$Mg^{2+}$] axis of this asymptote and the curve in the absence of enzyme will be the quantity of $Mg^{2+}$ bound by the enzyme at saturation. Hence the stoicheiometry of the association can be calculated. This can be checked by equilibrium dialysis.

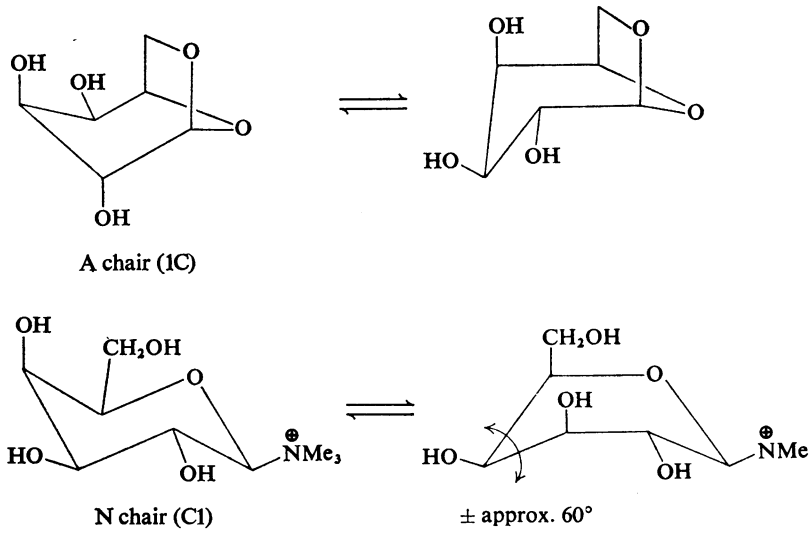


Fig. 1. *Possible conformations of* 1,6-*anhydro*-D-*galactopyranose and of the* β-D-*galactopyranosyl trimethylammonium ion*

Conformational distortion of the substrate is considered to make a substantial contribution to the catalytic efficacy of many enzymes (Jencks, 1969; Wolfenden, 1972). In the absence of an X-ray determination of the structure of an enzyme–inhibitor complex, gross conformational changes of the substrate on binding can be detected by the synthesis of potential substrates or inhibitors that have only a limited range of conformations available. β-D-Galactopyranosyl trimethylammonium bromide is such a compound: the trimethylammonium group is isoelectronic and isosteric with the t-butyl group, whose reluctance to adopt an axial position is well documented (Winstein & Holness, 1955; Campbell *et al.*, 1968). Further, the anomeric effect acting on the full positive charge on the nitrogen atom will also ensure that the C–N bond remains equatorial (cf. Lemieux & Morgan, 1965). A further compound that might throw light on the conformation in which the enzyme binds substrates and inhibitors is 1,6-anhydrogalactopyranose. The molecular structure of this compound precludes the adoption of the N chair (C1) conformation (Fig. 1); however, since the enzymic specificity for the substituent at C-5 of the pyranose ring is not great (Wallenfels & Malhotra, 1961), and the glycosidic linkage has the β configuration, the compound has all the requirements for a substrate, except for its limited range of possible conformations.

## Methods and Materials

### *Materials*

1,6-Anhydro-D-galactopyranose, m.p. 217°C (from EtOAc), and β-D-galactopyranosyl trimethylammonium bromide, m.p. 165–168°C (from EtOH), were made by the method of Micheel (1929), who gives m.p. 220–221°C and 162–164°C respectively. 4-Nitrophenyl β-D-galactopyranoside was made by the method of Csűrös *et al.* (1964), m.p. 176–177°C (lit. 177–178°C). Tetramethylammonium bromide (lot no. 2866310) was purchased from BDH Ltd., Poole, Dorset, U.K., and phenyl β-D-galactopyranoside (lot no. 47467) was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. β-D-Galactopyranose dehydrogenase (lot no. 7081513) was purchased from Boehringer Ltd., Uxbridge, Middx., U.K.

Techniques for the production and manipulation of $Mg^{2+}$-free β-galactosidase have been described (Tenu *et al.*, 1972); other β-galactosidase was purchased from Boehringer Ltd. (lot no. 7500408).

### *Testing of compounds*

*Substrate tests.* The technique of Wallenfels & Kurz (1963) was used to determine β-galactosidase-hydrolysable material. In 0.1 M-sodium phosphate buffer, pH 7.0, containing 1.0 mM-$MgCl_2$, at 25.0°C, there was no detectable increase in absorbance at

340nm of a solution containing $NAD^+$ (1.5mM), β-galactosidase (4μM), galactose dehydrogenase (0.05mg/ml) and 0.193M-1,6-anhydrogalactose or 0.193M-β-D-galactopyranosyl trimethylammonium bromide over 30min. Replacement of either of the potential substrates by the known substrate phenyl β-D-galactopyranoside (1.6mM), and decreasing the β-galactosidase concentration by an order of magnitude, gave a rapid absorbance increase of more than 2 units (1cm-light-path cell). At these concentrations of potential substrate the enzyme was saturated with β-D-galactopyranosyl trimethylammonium bromide, and an approximate maximum value of $k_{cat.}$ of $5\times10^{-3}s^{-1}$ was calculated. Since the 1,6-anhydrogalactopyranose did not appreciably inhibit the enzyme, a maximum value (of $3\times10^{-2}M^{-1}\cdot s^{-1}$) can be put only on $k_{cat.}/K_m$.

*Inhibitor tests.* Neither 20mM-tetramethylammonium bromide nor 20mM-1,6-anhydrogalactopyranose detectably inhibited the hydrolysis of 4-nitrophenyl β-D-galactopyranoside (21μM; $K_m = 30\mu M$) by β-galactosidase (2.5nM), at 25.0°C in the phosphate medium described, i.e. $K_i$ for both compounds > 0.2M. Strong inhibition was observed with β-D-galactopyranosyl trimethylammonium bromide; rough calculation on the assumption that the inhibition was competitive indicated $K_i = 1$mM.

*Determination of $K_i$*

*$K_i$ for β-D-galactopyranosyl trimethylammonium bromide.* An 8×7 array of inhibitor and substrate concentrations was used: 4-nitrophenyl β-D-galactopyranoside concentrations of 6, 12, 18, 24, 30, 39.2, 60 and 150μM were used with inhibitor concentrations of 0, 1.44, 2.96, 4.48, 6.00, 7.44 and 12.0mM. The initial rates of increase of $E_{400}$ were fitted directly to the expression for competitive inhibition by using a library program associated with a Hewlett–Packard 9100B calculator. All the foregoing measurements were performed on a Unicam SP.1800 spectrophotometer system, with a cell-block thermostatically maintained at 25.0±0.1°C by a Julabo Paratherm water-circulating pump. Appearance of 4-nitrophenol was followed at 400nm.

*$K_i$ for β-D-galactopyranosyl trimethylammonium bromide in the absence of $Mg^{2+}$.* These experiments were performed with a Cary 14M spectrophotometer system, with a cell-block thermostatically maintained at 25.0±0.1°C. The medium for the $K_i$ determination was 10mM-EDTA–NaOH, pH7.0, containing 0.145M-NaCl. Total enzyme concentration was 0.02μM (protomer), and substrate concentrations ranged from 90μM to 10mM; the substrate could in this case be 2-nitrophenyl galactoside, since in the absence of $Mg^{2+}$ degalactosylation is no longer partially rate-limiting, with its consequent potential complication of uncompetitive inhibition (Viratelle & Yon, 1973).

*Experiments relating to the slow loss of $Mg^{2+}$ from enzyme*

The increase in $E_{405}$ consequent on the addition of 0.50μM-enzyme (protomer) solution, 0.145M in NaCl (20μl), to a solution of 10mM-2-nitrophenyl β-D-galactopyranoside, 0.145M in NaCl (3.0ml), in 10mm-light-path cells in the cell compartment (thermostatically maintained at 25.0°C) of a Cary 14M spectrophotometer, was recorded. For curve (1) (Fig. 3) the substrate solution contained 1.0mM-$MgSO_4$ and was buffered to pH7.0 with 10mM-*N*-(ethyl-2′-hydroxysulphonyl)-2-amino-2-hydroxymethylpropane-1,3-diol–NaOH (buffer A); otherwise the solution was buffered to pH7.0 by 10mM-EDTA–NaOH and contained no $Mg^{2+}$. The enzyme solutions for curves (1), (2) and (3) contained buffer A (10mM), pH7.0, and respectively 1.0mM-$MgSO_4$, 20μM-$MgSO_4$ and 10.7mM-β-D-galactopyranosyl trimethylammonium bromide, and 20μM-$MgSO_4$ alone. The enzyme solutions for curves (4) and (5) contained 10mM-EDTA–NaOH at pH7.0, and only the former contained 10.7mM-β-D-galactopyranosyl trimethylammonium bromide.

*Other techniques*

*Fluorescence measurements.* These were performed in an Aminco–Bowman spectrofluorimeter with an exciting wavelength of 385nm; the emission maximum of 8-hydroxyquinoline-5-sulphonic acid is 495nm. Metal-free enzyme was produced by dialysis of 11μM-enzyme protomer solutions against 2mM-8-hydroxyquinoline-5-sulphonic acid, 0.145M in NaCl, at pH8.0.

*Equilibrium dialysis.* Polythene apparatus was used throughout. A solution of enzyme (approx. 3mg/ml) in 0.1M-Tris–acetate buffer, pH7.0, 1.0mM in $MgSO_4$ (1ml), was dialysed at 4°C against 2×500ml of 1mM buffer A, pH7.0, containing 0.145M-NaCl, to remove loosely bound ions. After equilibration $Mg^{2+}$ in the diffusate was determined by its atomic absorption line at 285.2nm by using Perkin–Elmer 290B equipment, and its concentration was adjusted to 5μM. Dialysis was continued at 22°C for 12h, the dialysis bag was removed, and denatured protein centrifuged off the dialysis residue. Undenatured protein was estimated from its u.v. absorbance at 280nm by assuming a molecular extinction coefficient of $2.83\times10^5M^{-1}\cdot cm^{-1}$. The pH was then adjusted to 5.1 with 0.1M-acetic acid (10μl), and the difference between the $Mg^{2+}$ concentration of the sample and of a similarly treated sample of the dialysis buffer was measured by atomic absorption.

## Results and Discussion

Potential inhibitors were tested against 4-nitrophenyl β-D-galactopyranoside, since for this substrate some step before degalactosylation is rate-limiting (Tenu *et al.*, 1971), and binding to the acceptor site should not present problems.

1,6-Anhydrogalactopyranose does not significantly bind to the enzyme ($K_i > 0.2$M). β-D-Galactopyranosyl trimethylammonium bromide was a good competitive inhibitor: analysis of a 8×7 array of inhibitor and 4-nitrophenyl β-D-galactopyranoside concentrations indicated $K_i = 1.4 \pm 0.2$mM, $K_m = 31 \pm 2\mu$M, $V_{max.} = 0.52 \pm 0.02 \mu M \cdot s^{-1}$ ($[E]_0 = 5$nM, approx. 60% active). The correspondence of the $K_m$ value with that obtained by Tenu *et al.* (1971), and the low error on $V_{max.}$, confirm that the inhibition is competitive. Although amine cations are known to inhibit the enzyme (Kuby & Lardy, 1953), the lack of inhibition by tetramethylammonium bromide confirms that this is not the cause of the inhibition by the galactosyl derivative.

A β-D-galactopyranose derivative that cannot adopt the C1 chair conformation (1,6-anhydrogalactopyranose) shows no sign of interacting with the enzyme at all: a β-D-galactopyranose derivative that cannot adopt anything but the C1 conformation and a limited range of 'twist-boat' conformers is a moderately good competitive inhibitor. We can therefore conclude that the enzyme first binds the substrate in some approximation to the C1 chair conformation, since adoption of the relatively limited range of boat conformations allowed by the structure of the cationic inhibitor would not facilitate loss of aglycone, and would be energetically unfavourable. However, lesser conformational distortion of the type postulated to occur in lysozyme-catalysed hydrolyses (cf. Chipman & Sharon, 1969) is compatible with this result.

It has been postulated (Sinnott, 1971) that all substrates of β-galactosidase must possess a lone pair of electrons on the atom directly attached to C-1 of the pyranose ring. The acidic group must co-ordinate to this lone pair to remove the aglycone. β-Galactopyranosyl trimethylammonium bromide thus presents all the features of a substrate (p$K_a$ of trimethylamine = 9.8), except this, and is not a substrate

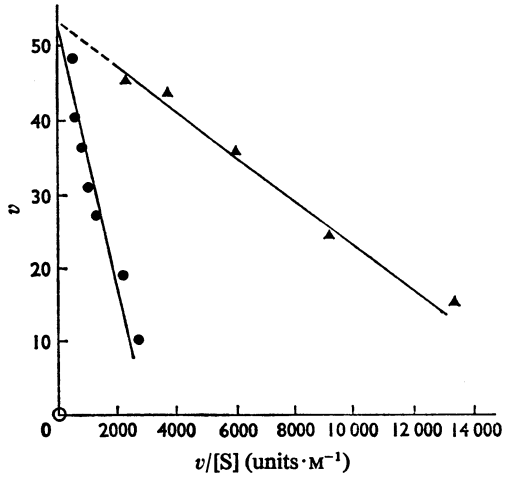


Fig. 2. *Inhibition of $Mg^{2+}$-free β-galactosidase by β-D-galactopyranosyl trimethylammonium bromide*

Velocities ($v$, in arbitrary units) of hydrolysis of 2-nitrophenyl β-D-galactopyranoside (in 10mM-EDTA–NaOH, pH7.0, 0.145M in NaCl, at 25°C) in the presence (●) and the absence (▲) of 10.7mM-β-D-galactopyranosyl trimethylammonium bromide were measured. For details see the Methods and Materials section.

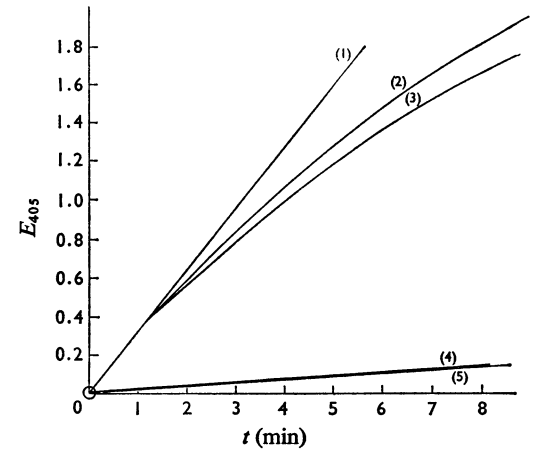


Fig. 3. *Deactivation of β-galactosidase in 10mM-EDTA*

The curves show the extinction of a 10mM solution of 2-nitrophenyl β-D-galactopyranoside (10mm light-path cell) after the addition of 3.3nM-enzyme protomer (at 25.0°C). (1) Medium buffer A (10mM), pH7.0, 1.0mM-$MgSO_4$; enzyme preincubated with $Mg^{2+}$. (2) Medium 10mM-EDTA–NaOH, pH7.0; enzyme preincubated with 20μM-$MgSO_4$. (3) Medium 10mM-EDTA–NaOH, pH7.0; enzyme preincubated with 20μM-$MgSO_4$ and 10.7mM-β-D-galactopyranosyl trimethylammonium bromide. (4) Medium 10mM-EDTA–NaOH, pH7.0; enzyme preincubated in the same medium. (5) Medium 10mM-EDTA–NaOH, pH7.0; enzyme preincubated in the same medium containing 10.7mM-β-D-galactopyranosyl trimethylammonium bromide. For further details see the Methods and Materials section.

($k_{cat.} < 5 \times 10^{-3} s^{-1}$). This confirms the original hypothesis.

The intuitive expectation is that the full positive charge on the cationic inhibitor would prevent its binding to an active site that was positively charged. This expectation can be quantified if an appropriate uncharged model can be found: an ideal model would be 2-(β-D-galactopyranosyl)-2-methylpropane, which is isosteric and isoelectronic with the cationic species. Since the obvious approach to its synthesis (2-methyl-2-propylzinc bromide on 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide) fails (D. Cocker & M. L. Sinnott, unpublished work), alternative approaches would represent synthetic undertakings of considerable magnitude. $K_i$ for methyl 1-thio-β-D-galactopyranoside is 1.8mM (Sinnott, 1971), $K_m$ for methyl β-D-galactopyranoside is 8mM (Tenu *et al.*, 1972), $K_i$ for galactose is 4mM (O. M. Viratelle, unpublished work), and therefore it seems reasonable to assume that the $K_i$ for an uncharged model for β-D-galactopyranosyl trimethylammonium bromide would be in the range 1–10mM. Therefore it seems likely that the active site is not positively charged. This seems compatible with a role for $Mg^{2+}$ as an electrophile only if binding of this ion and β-D-galactopyranosyl trimethylammonium bromide is competitive.

Competitive binding of $Mg^{2+}$ and β-D-galactopyranosyl trimethylammonium bromide is ruled out by two lines of evidence.

(1) The $K_i$ value for β-D-galactopyranosyl trimethylammonium bromide in the absence of $Mg^{2+}$ (i.e. in 10mM-EDTA) is approx. 2.2mM. Plots of $v$ against $v/[S]$ (Fig. 2) clearly indicate that the inhibition is still competitive. However, prolonged incubation of the inhibitor with $Mg^{2+}$-free enzyme caused irreversible denaturation, although $Mg^{2+}$-saturated enzyme was stable indefinitely in the presence of 20mM quaternary ammonium salt. The $K_i$ value for β-D-galactopyranosyl trimethylammonium bromide in the absence of $Mg^{2+}$ (2.2mM) is not in very good agreement with that in its presence (1.4mM), but the discrepancy is probably due to secondary effects (e.g. change of medium) and is in any event in the wrong direction for competitive binding of $Mg^{2+}$ and inhibitor.

(2) The uptake of $Mg^{2+}$ by $Mg^{2+}$-free enzyme, and its loss in EDTA, are both slow (Tenu *et al.*, 1972). If binding of the inhibitor is competitive with binding of $Mg^{2+}$, then saturation of the enzyme with β-D-galactopyranosyl trimethylammonium bromide in an $Mg^{2+}$-rich medium should nonetheless result in an $Mg^{2+}$-free enzyme. If the enzyme were preincubated with this salt, and then placed in a $Mg^{2+}$-scavenging medium (10mM-EDTA), no slow deactivation should

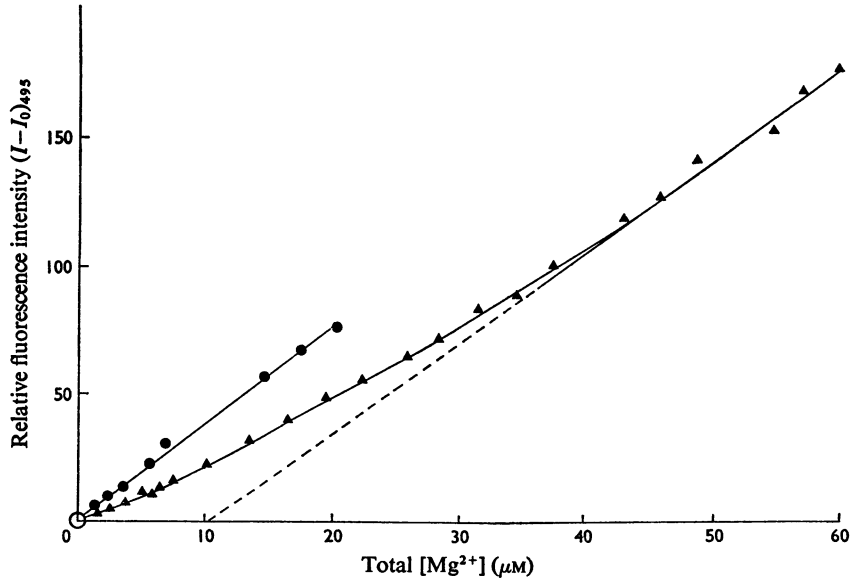


Fig. 4. *Stoicheiometry of $Mg^{2+}$ binding*

The reaction was studied at pH8.0 and 22°C. β-Galactosidase protomer concentration: ●, 0; ▲, 11μM. 8-Hydroxyquinoline-5-sulphonic acid concentration: 2.0mM. For details see the Methods and Materials section.

be observed; the enzyme should have the activity of $Mg^{2+}$-free enzyme. Fig. 3 shows that this is not the case; curves show the change in extinction on addition of variously treated enzyme to 10mM-2-nitrophenyl β-D-galactopyranoside. Curve (1) is obtained in the absence of EDTA, the others in its presence. Curve (2) is obtained with enzyme pre-incubated with both β-D-galactopyranosyl trimethylammonium bromide and $Mg^{2+}$, curve (3) with $Mg^{2+}$ alone, curve (4) with quaternary salt alone and curve (5) with $Mg^{2+}$-free enzyme. The similarity of curves (2) and (3) indicate that binding of this salt does not remove $Mg^{2+}$; the similarity of curves (4) and (5) indicates that loss of β-D-galactopyranosyl trimethylammonium bromide from the enzyme is fast compared with the processes that we are observing.

Therefore the binding of the quaternary salt and $Mg^{2+}$ is completely non-competitive. A function for the $Mg^{2+}$ as an electrophilic catalyst in the generation of a galactopyranosyl cation can therefore be ruled out. The $Mg^{2+}$ probably acts to stabilize a favourable protein conformation; this is in any event made likely by the retention of some activity in the $Mg^{2+}$-free enzyme.

The fluorescence of 8-hydroxyquinoline-5-sulphonic acid in the presence of $Mg^{2+}$ alone and in the presence of $Mg^{2+}$ and enzyme (Fig. 4) indicates that 1 $Mg^{2+}$ ion is bound per 135000-dalton protomer. Equilibrium-dialysis measurements were made with low concentrations of $Mg^{2+}$ (5μM); the apparent dissociation constant of the $Mg^{2+}$–enzyme complex at pH7.0 is 0.65μM (Tenu *et al.*, 1972). Therefore, although enzyme will be largely saturated with $Mg^{2+}$ at its tightest binding site, insufficient $Mg^{2+}$ will be used to saturate any non-specific sites. Under these conditions we find that 1.1 $Mg^{2+}$ ions are bound per protomer. The $Mg^{2+}$ ion appears thus to play a much simpler role than the $Zn^{2+}$ ion in another hydrolase, alkaline phosphatase, from the same micro-organism (Halford *et al.*, 1972).

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## References

Campbell, N. C. G., Muir, D. M., Hill, R. R., Parish, J. H. & Whiting, M. C. (1968) *J. Chem. Soc. B* 355–364

Chipman, D. M. & Sharon, N. (1969) *Science* **165**, 454–465

Clark, C. R., Hay, R. W. & Dea, I. C. M. (1970) *Chem. Commun.* 794–795

Csűrös, Z., Deák, Gy. & Haraszthy-Papp, M. (1964) *Acta Chim. (Budapest)* **42**, 263–267

Dawson, R. M. C. (1969) *Data for Biochemical Research*, pp. 428–429, Clarendon, Oxford

Halford, S. E., Schlesinger, M. J. & Gutfreund, H. (1972) *Biochem. J.* **126**, 1081–1090

Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp. 282–322, McGraw-Hill, New York

Kuby, S. A. & Lardy, H. A. (1953) *J. Amer. Chem. Soc.* **75**, 890–896

Lemieux, R. U. & Morgan, A. R. (1965) *Can. J. Chem.* **43**, 2205–2213

Micheel, F. (1929) *Chem. Ber.* **62**, 687–693

Petroský, V. (1966) *Coll. Czech. Chem. Commun.* **32**, 2656–2660

Sinnott, M. L. (1971) *Biochem. J.* **125**, 717–719

Sinnott, M. L. & Souchard, I. J. L. (1973) *Biochem. J.* **133**, 89–98

Tenu, J. P., Viratelle, O. M., Garnier, J. & Yon, J. (1971) *Eur. J. Biochem.* **20**, 363–370

Tenu, J. P., Viratelle, O. M. & Yon, J. (1972) *Eur. J. Biochem.* **26**, 112–118

Viratelle, O. M. & Yon, J. (1973) *Eur. J. Biochem.* in the press

Wallenfels, K. & Kurz, G. (1963) *Biochem. Z.* **335**, 559–572

Wallenfels, K. & Malhotra, O. P. (1961) *Advan. Carbohyd. Chem.* **16**, 239–298

Winstein, S. & Holness, N. J. (1955) *J. Amer. Chem. Soc.* **77**, 5562–5578

Wolfenden, R. (1972) *Accounts Chem. Res.* **5**, 10–18